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Discovering the Sequence Specificity of Human DYRK2 and DYRK4

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Discovering the Sequence Specificity of Human DYRK2 and DYRK4

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University Scholar Project

May 2015

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I. Abstract

Protein phosphorylation is a post-translational modification (PTM) that is ubiquitous in regulating cellular processes. It is the most common PTM used in signal translation.

Protein kinases are the class of enzymes that catalyze the transfer of a phosphate group from ATP to a specific amino acid on a substrate protein. In eukaryotes, kinases generally add a phosphate to serine, threonine, or tyrosine residues. Short linear patterns in the amino acid sequence of the substrate protein help guide the protein kinase to the correct residue to be phosphorylated. However, these patterns, or “motifs,” as well as the complete list of substrates for each kinase are largely unknown. A method known as ProPeL (Proteomic Peptide Library) can be utilized to discover motifs, and thus potential target substrates, of a protein kinase-of-interest. For this project, ProPeL and the visualization tool known as pLogo (Probability Logo Generator) have been utilized to examine the specificity motifs of two DYRK family protein kinases, DYRK2 and DYRK4. Our preliminary data suggests that the substrate specificities for DYRK2 and DYRK4 are similar to the canonical member, DYRK1a; however, distinct differences have been found. For example, DYRK2 may have a higher stoichiometry of protein phosphorylation at tyrosine sites, and DYRK4 may not

exhibit the typical preference for serine over threonine when phosphorylating substrates. For both DYRK2 and DYRK4, additional data will need to be collected to obtain more statistically significant results.

II. Introduction

Protein Phosphorylation

Post-translational modifications (PTMs) are found on almost every protein and these modifications have been found to be fundamental for cellular regulation.¹ Reversible protein phosphorylation is one of the most common PTMs in eukaryotic cells.² Reversible phosphorylation comprises two opposing reactions: phosphorylation and dephosphorylation. Phosphorylation is catalyzed by a class of enzymes, called protein kinases, and dephosphorylation is catalyzed by a class of enzymes, called phosphatases.^{1,3} Protein kinases and phosphatases can alter the function of a protein in a multitude of ways. For example, adding or removing a phosphate group from a protein can increase or decrease its biological activity, stabilize or mark the protein for degradation, or facilitate or disrupt the formation of protein-protein interactions.^{4,5}

The mechanism of protein phosphorylation by a protein kinase involves the transfer of the γ -phosphate group of ATP to the hydroxyl oxygen of a serine, threonine, or tyrosine residue of a substrate (**Figure 1**).^{1,5}

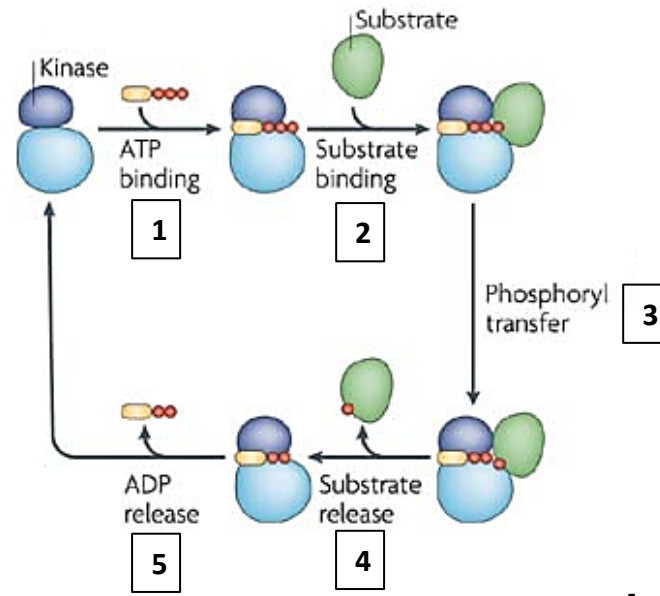


Figure 1. Mechanism of substrate phosphorylation by a protein kinase.⁵ (1) ATP binds to the active site of a kinase. (2) The substrate binds to the active site. (3) The gamma-phosphate of ATP is transferred to a serine, threonine, or tyrosine residue of the substrate. (4) Following phosphorylation, the substrate is released from the kinase. (5) ADP is released from the active site of the kinase. This figure is adapted from Ubersax *et al.* (2007).⁵

It is estimated that one-third of all of the proteins encoded by the human genome are modified by phosphorylation.^{4,5} Therefore, it is not surprising that protein phosphorylation is crucial for regulating numerous cellular processes, such as metabolism, cell proliferation, and apoptosis.^{1,3} Abnormal phosphorylation can also be the cause or symptom of many human diseases, such as cancer.^{6,7}

Protein Kinase Specificity

Currently, there are over 250,000 annotated phosphorylation sites in mammals and every kinase varies greatly in its expression, activity, and function.⁸ Therefore, there must be a mechanism for distinguishing specific substrate(s) among the thousands of proteins. It has been determined that most kinases are directed to their substrate, commonly through temporal and spatial co-expression and/or protein-protein interactions.⁵ Once a kinase is located near its substrate, short linear patterns or

“motifs” in the primary structure of the substrate guide the catalytic pocket of the kinase to the correct residue to be phosphorylated.⁹ Often, the substrate motif and the complementary sequence of the kinase interact through ionic bonding, hydrogen bonding, or hydrophobic interactions, which increase the local substrate specificity of many kinases. Identifying the specificity motif for a kinase can be a useful first step toward pinpointing its downstream target substrates and its overall function in the cell.^{5,9,10,11}

ProPeL Methodology

In order to determine the specificity motif for a kinase, the sites on a protein that the kinase has phosphorylated must be found. Once found, the phosphorylated sites, or phosphopeptides, can be analyzed for patterns. These patterns can then be used to find the specificity motif of a kinase.^{9,11}

A new method, developed by the Schwartz Lab at the University of Connecticut, to determine the specificity motif of protein kinases is called ProPeL (Proteomic Peptide Library, **Figure 2**).¹³

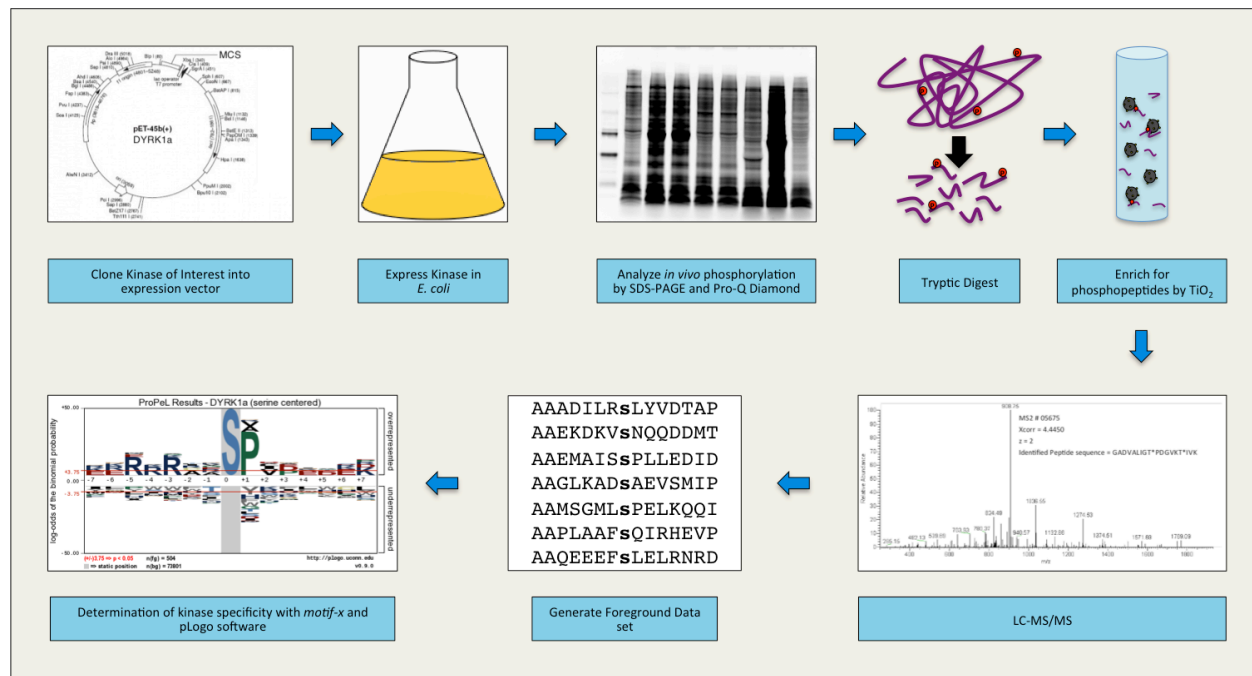


Figure 2. A general overview of ProPeL methodology.^{13,15} The gene for a kinase-of-interest is cloned into a bacterial expression vector (i.e. pET45b). Next, transformed *E. coli* cells are induced to overexpress the kinase. To analyze *in vivo* phosphorylation activity of the kinase, SDS-PAGE is performed and the protein gels are stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies). After confirming kinase activity, the cell lysate containing the kinase and phosphopeptides is purified and the proteins are cleaved by trypsin. Titanium Dioxide (TiO₂) is implemented to enrich the sample for phosphopeptides. Next, the sample is analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). A foreground data set is generated by mapping the unique phosphorylated tryptic peptides that were identified by LC-MS/MS. Each phosphorylated 15-mer is centered on a phosphorylation site (i.e. serine). The data set is inputted into the pLogo software to determine the specificity of the kinase-of-interest. This figure is adapted from Chou *et al.* (2012).¹³

ProPeL uses bacteria, such as *E. coli*, to function as an *in vivo* peptide library for thousands of simultaneous phosphorylation reactions carried out by a protein kinase-of-interest. This strategy begins with the cloning and expression of the kinase of interest in *E. coli* competent cells. *E. coli* is a convenient model organism for this strategy because it does not encode any eukaryotic-like serine/threonine kinases and has very low levels of endogenous serine and threonine phosphorylation. This ensures that the majority of detected phosphopeptides will be the result of phosphorylation by the expressed kinase.¹³

After the kinase is successfully expressed and active, the kinase phosphorylates the bacterium's proteome *in vivo*, in a manner that is consistent with its sequence specificity. After expression, the bacterial proteins are extracted from the cell lysate and digested by trypsin.^{9,12,13} Trypsin is used because the distribution of arginines and lysines in the bacterial proteome is such that the resulting peptides are usually 5-15 amino acids long, which is close to the size needed for standard bottom-up mass spectrometry workflows (~7 to 35 amino acids long).^{9,11}

The digested proteins are purified and enriched for phosphopeptides using titanium dioxide. Next, liquid chromatography tandem-mass spectrometry (LC-MS/MS) is utilized to determine the sequence of the tryptic phosphopeptides.¹⁴ Using these sequences and pLogo (Probability Logo Generator), the specificity motif for the kinase-of-interest can be determined.^{13,15}

pLogo Visualization Tool

pLogo is a graphical representation of amino acid preferences at each position in a sequence motif (**Figure 3**).¹⁵ pLogos illustrate residues proportional to the log-odds of their binomial probability with respect to a certain background. For ProPeL pLogos, foreground data is obtained by mapping and extending tryptic peptides from LC-MS/MS to the *E. coli* proteome. Background data is generated by aligning all of the unique serine-, threonine-, or tyrosine-centered 15-mers in the *E. coli* proteome.¹³ These 15 residue-long peptides are generated by the peptidextender tool, which is a peptide extension web tool found at <http://schwartzlab.uconn.edu/pepextend/>. The pLogo generation tool is available for use by the public at <http://plogo.uconn.edu/>.

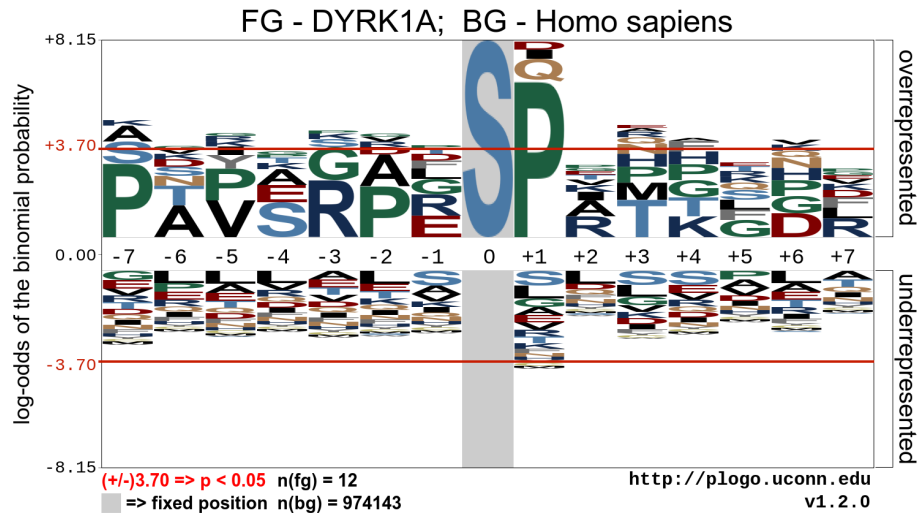


Figure 3. Visualizing sequence specificity using pLogo.¹⁵ Each pLogo image has the following characteristics. (1) The height of a residue is proportional to its statistical significance. (2) Over- and under-represented residues are portrayed, above and below the x-axis, respectively. (3) The y-axis represents the odds of binomial probability on a logarithmic scale. The horizontal lines represent the Bonferroni-corrected statistical significance values. (4) Residues are stacked in order of their statistical significance; the most significant residues are positioned closest to the x-axis. (5) Fixed motif positions are highlighted in gray. This figure is adapted from O'Shea *et al.* (2013).¹⁵

The DYRK Family of Protein Kinases

General Features. The dual-specificity tyrosine-phosphorylation-regulated kinases (DYRKs) are evolutionary conserved enzymes belonging to the CMGC group of protein kinases.^{16,17,18} There are five human DYRKs: DYRK1a, DYRK1b, DYRK2, DYRK3, and DYRK4 (**Figure 4**).^{19,20} Each DYRK contains a conserved kinase domain that has a characteristic structure. Adjacent to the kinase domain is the DYRK homology box, which contains variable N- and C-terminal regions. These regions are different in each DYRK and may be involved in the regulation of DYRK kinase activity. All DYRKs have been found to autophosphorylate on the second tyrosine residue of the YXY motif in the activation loop, which is found in the catalytic domain.

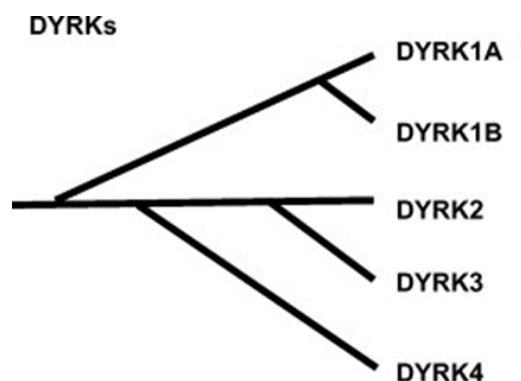


Figure 4. A phylogenetic tree of human DYRK kinases. This figure is adapted from Yoshida (2008).²⁰

In addition, DYRKs are known to phosphorylate substrates on serine and/or threonine residues.^{17,20,21} It has been suggested that DYRKs autophosphorylate a tyrosine residue, but they only phosphorylate their substrates on serine/threonine residues.²¹ Members of the DYRK family are involved in regulating key developmental and cellular processes such as neurogenesis, cell growth, the cell cycle, and cellular differentiation. Even though DYRK kinases are critical for cell function, the exact substrate specificity of each DYRK for serine/threonine phosphorylation has not been determined.^{16,18,25} It has only been suggested that, based on sequence homology in the conserved kinase domain, DYRK kinases have a preference for upstream basic residues and a proline in the +1 position when phosphorylating substrates.¹⁸ This project is focused on the type II DYRKs (DYRK2, DYRK3, and DYRK4), however, we do not have results for DYRK3 yet.

DYRK2. DYRK2 is involved in the same cellular and developmental processes as the other DYRKs, but its specific cellular functions are only beginning to be discovered.¹⁹ Recent studies have indicated that DYRK2 may have a significant role in tumor development and/or progression.^{20, 21} There is evidence that DYRK2 translocates from the cytoplasm to the nucleus following DNA damage. Once in the nucleus, DYRK2 can

phosphorylate p53 at Ser⁴⁶, which induces apoptotic cell death.²⁰ However, even though there is significant evidence that DYRK2 is involved in tumor growth and metastasis, the substrate specificity for this kinase is still unknown.^{19,20,21} Discovering the substrate specificity of DYRK2 is essential for understanding the functional role of DYRK2 in the cell and in human disease.

DYRK4. The least studied member of the DYRK family is DYRK4. Besides its raw sequence, there is hardly any information about its function or its possible target substrates.²⁰ It has been suggested that DYRK4 is a testis-specific kinase with a possible role in spermatogenesis^{20,24}; however, other researchers have proposed that different splice variants of DYRK4 are expressed in tissue-specific patterns.^{20,24,25} More research is needed to determine the functional role and substrate specificity of DYRK4.

Thesis Project

To determine the specificity motif and to compare the substrate specificities of DYRK2 and DYRK4, ProPeL and pLogo were utilized in this project.^{13,15} The preliminary data suggests that the substrate specificities for DYRK2 and DYRK4 are similar to those typical of DYRK members. However, there are unique differences between them. DYRK2 appears to be able to phosphorylate tyrosine substrates and DYRK4 seems to not prefer serine to threonine substrates. With more data, the substrate specificities of DYRK2 and DYRK4 will be conclusively determined.

III. Results

In vivo Phosphorylation Analysis

To prove that DYRK2 and DYRK4 were expressed and active in *E. coli*, total protein samples from cell lysates expressing either DYRK2 or DYRK4 were separated

by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and stained sequentially with Pro-Q Diamond gel stain (**Figure 5A**) and GelCode Blue gel stain (**Figure 5B**). Pro-Q Diamond gel stain was used to detect phosphorylated peptides. However, Pro-Q Diamond stain also has non-specific activity. GelCode Blue staining, which is analogous to Coomassie total protein staining, was used to ensure that differences in Pro-Q Diamond staining were not due to gel loading differences. Since the negative control (lysate with an empty expression vector) will still contain endogenously phosphorylated *E. coli* proteins, phosphorylation levels related to kinase activity must be compared to the negative control.

The representative gel, containing the samples used for mass spectrometric identification of phosphopeptides, is shown in **Figure 5**. Other gels and expression conditions can be found in *Supplementary Materials*. Compared to the phosphorylation levels in *E. coli* expressing the empty pET45b vector (the negative control sample), the DYRK2 sample showed significantly higher levels of phosphorylation. The proteins in the DYRK4 sample did not show significantly higher levels of phosphorylation (**Figure 5A**). The GelCode Blue Stain shows that there are relatively equal amounts of protein in the negative control, DYRK2 sample, and DYRK4 sample (**Figure 5B**). Therefore, the differences in Pro-Q Diamond staining between samples were not the result of gel loading differences.

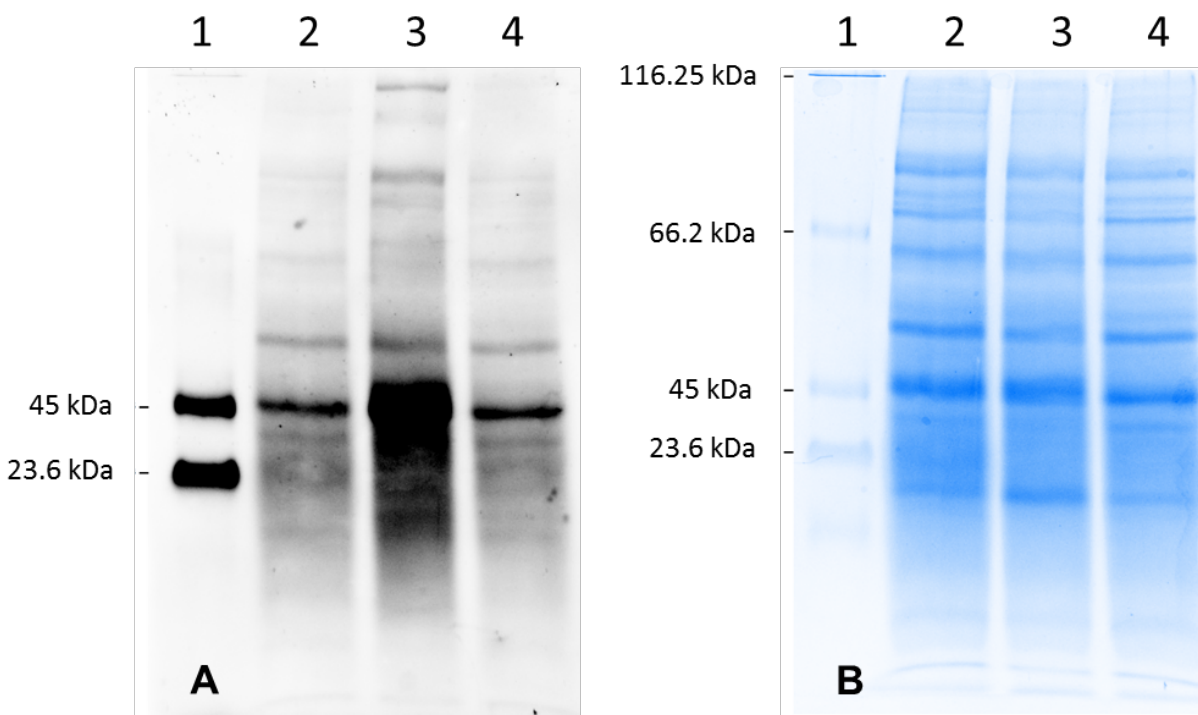
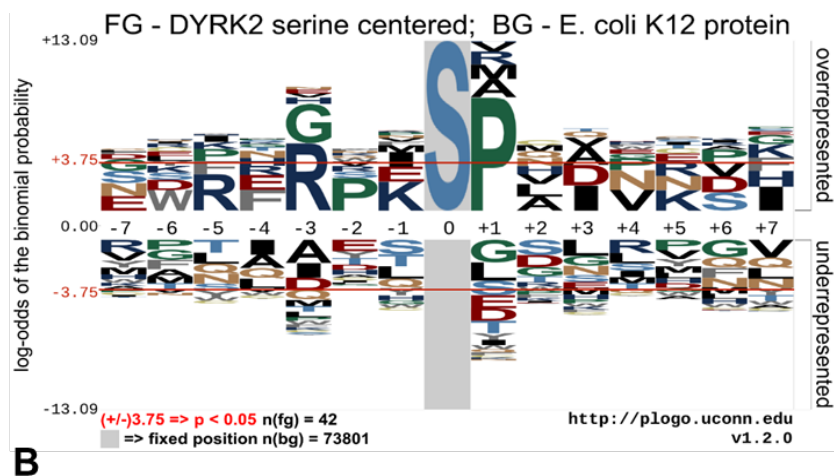
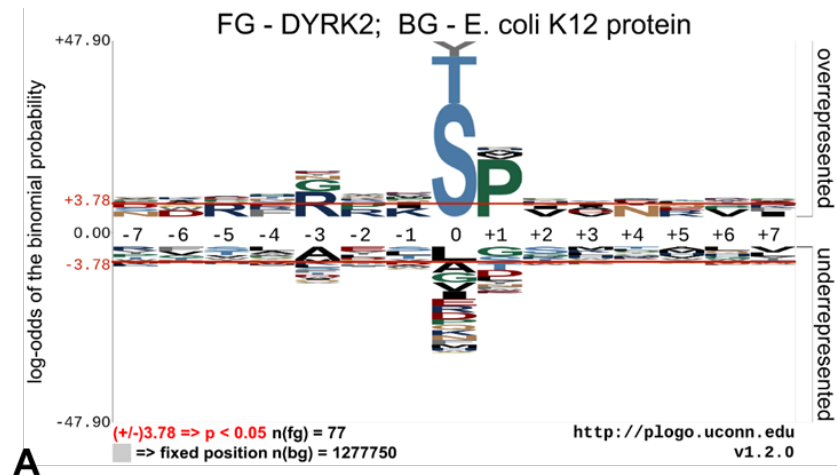


Figure 5. Analysis of *in vivo* phosphorylation. The protein stains utilized in this analysis are Pro-Q® Diamond Stain (Life Technologies) and GelCode™ Blue Safe Protein Stain (Life Technologies). Pro-Q Diamond gel stain specifically stains phosphoproteins in acrylamide gels. GelCode Blue staining is analogous to Coomassie total protein staining. (A and B) Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies) and GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific). Legend: Lane 1: PeppermintStick™ Phosphoprotein Ladder (Life Technologies), Lane 2: pET45b (Empty Vector Control), Lane 3: DYRK2, and Lane 4: DYRK4.

Preliminary DYRK2 Specificity

After applying the ProPeL methodology to the catalytic domain of DYRK2, endogenous phosphorylation sites obtained from the negative control, *E. coli* expressing the empty pET45b vector, were subtracted from the phosphorylation sites obtained from *E. coli* expressing the catalytic domain of DYRK2.^{13,26,27} Thus, the ProPeL methodology resulted in the detection of 77 unique phosphorylation sites in *E. coli* expressing the catalytic domain of DYRK2. **Figure 6** shows four DYRK2 pLogos with the ProPeL generated *E. coli* phosphopeptides as the foreground and the *E. coli* K-12 proteome as a background centered on zero residues (**Figure 6A**), fixed on serine (**Figure 6B**), threonine (**Figure 6C**), or tyrosine (**Figure 6D**). Based on the pLogo in **Figure 6A**, it

appears that DYRK2 is capable of phosphorylating serine, threonine, and tyrosine substrates. **Figures 6B** and **6C** suggest that for serine and threonine substrates, there is a preference for a proline in the +1 position and upstream basic residues. For tyrosine substrates, there appears to be a loss in the preference for +1 proline and upstream basic residues (**Figure 6D**).



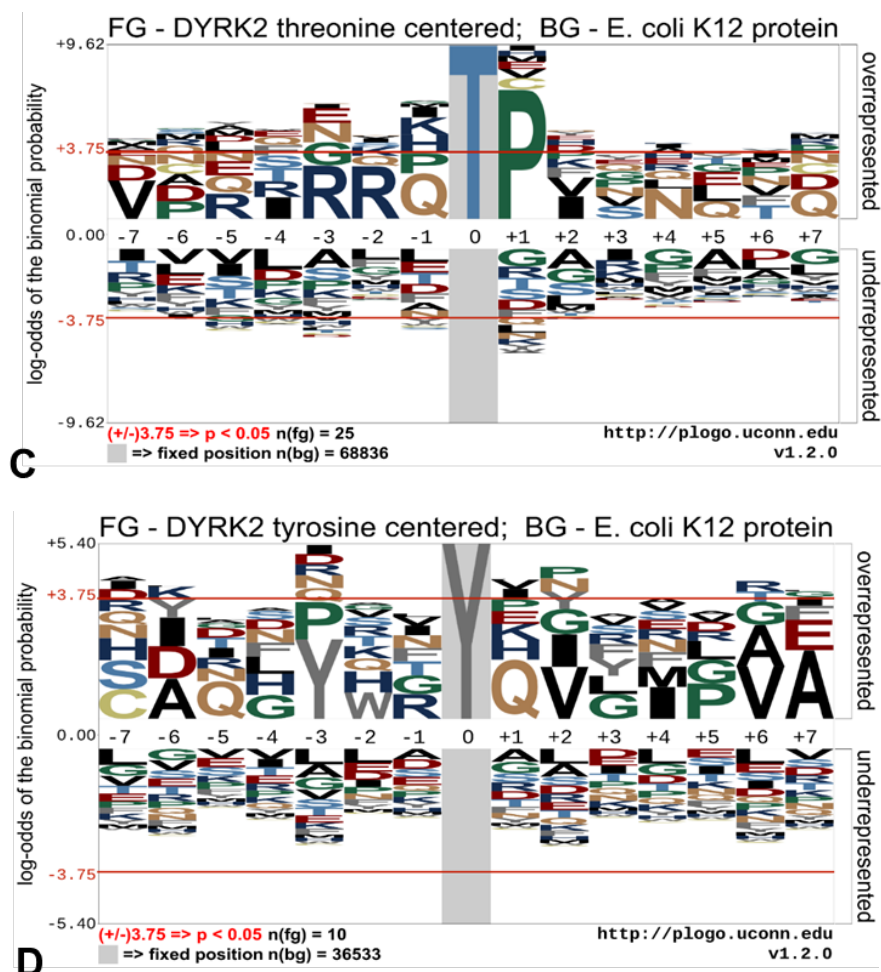
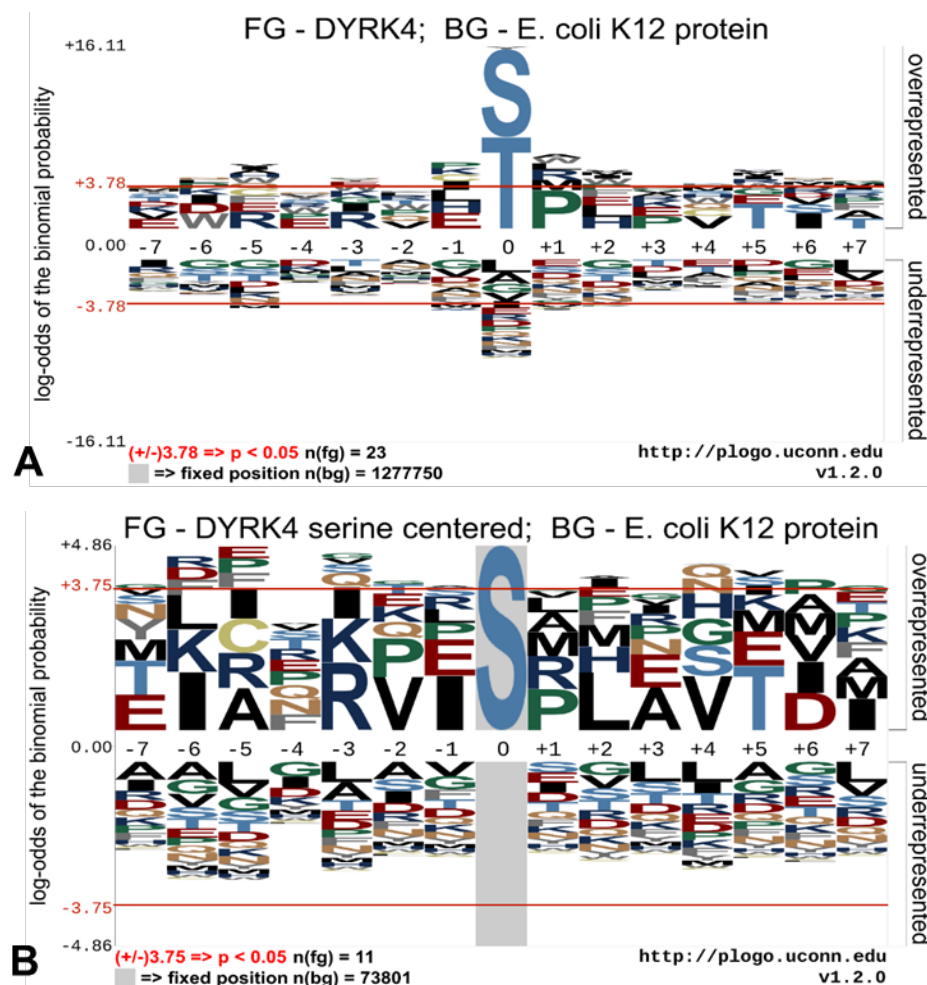


Figure 6. Preliminary DYRK2 substrate specificity. pLogos are constructed from phosphorylation sites in *E. coli*, which were obtained using the ProPeL methodology. (A) DYRK2 pLogo without any fixed residues. (B, C, and D) In each pLogo, the central residue is fixed. The fixed residue denotes the modification sites for (B) serine-centered, (C) threonine-centered, and (D) tyrosine centered preliminary specificity motifs for DYRK2.

Preliminary DYRK4 Specificity

After applying the ProPeL methodology to the catalytic domain of DYRK4, endogenous phosphorylation sites obtained from the negative control, *E. coli* expressing the empty pET45b vector, were subtracted from the phosphorylation sites obtained from *E. coli* expressing the catalytic domain of DYRK4.^{13,26,27} Thus, the ProPeL methodology resulted in the detection of 23 unique phosphorylation sites in *E. coli* expressing the catalytic domain of DYRK4. **Figure 7** shows three DYRK4 pLogos with the ProPeL

generated *E. coli* tryptic peptides as the foreground and the *E. coli* K-12 proteome as a background centered on zero residues (**Figure 7A**), serine (**Figure 7B**), or threonine (**Figure 7C**). All three DYRK4 plogos in **Figure 7** suggest that DYRK4 does not seem to have a strong preference for upstream basic residues and/or a proline in the +1 position when phosphorylating substrates. The DYRK4 pLogo shown in **Figure 7A** suggests that DYRK4 does not favor serine substrates over threonine substrates, and DYRK4 does not appear to be able to phosphorylate tyrosine substrates.



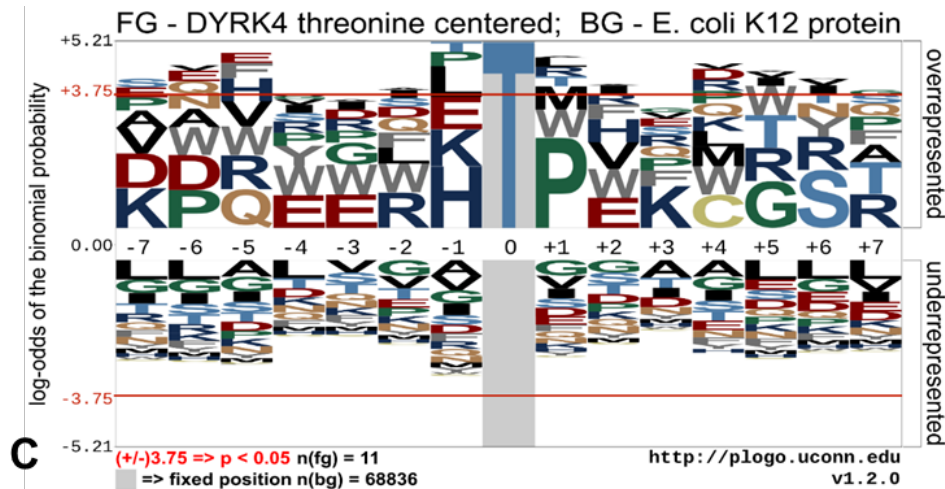


Figure 7. Preliminary DYRK4 substrate specificity. pLogos are constructed from phosphorylation sites in *E. coli*, which were obtained using the ProPeL methodology. (A) DYRK4 pLogo without any fixed residues. (B and C) In each pLogo, the central residue is fixed. The fixed residue denotes the modification sites for (B) serine-centered and (C) threonine-centered preliminary specificity motifs for DYRK4.

IV. Discussion

By expressing the catalytic truncation of DYRK2 and DYRK4 and analyzing the unique phosphorylation sites generated by ProPeL, I have been able to obtain preliminary data regarding the substrate specificity of these two kinases. Based on the literature and our lab's unpublished data, it has been indicated that the well-classified DYRK1a exhibits a strong preference for upstream basic amino acids, as well as a proline at the +1 position.²⁸ Therefore, it was exciting to find agreement with our data and previous research that for serine or threonine substrates of DYRK2, there appears to be a preference a proline in the +1 position. In addition, there seems to be an overall upstream basic residue preference, which is expected for members of the DYRK family. Interestingly, my data suggests that DYRK2 may exhibit a higher affinity for tyrosine substrates than other DYRK members.¹⁹ For DYRK2 tyrosine substrates, the preference for proline in the +1 position and the presence of upstream basic residues appear to be lost.

For DYRK4, the preliminary data suggests that there is a relative importance shift in the phosphorylation sites for DYRK4. For other DYRK family members, serine substrates are favored over threonine or tyrosine substrates. However, the amount of serine and threonine substrates for DYRK4 is the same. In addition, there appears to be less preference for upstream basic residues and a proline in the +1 position for DYRK4 substrates. Unfortunately, compared to DYRK2, fewer phosphopeptides were identified for DYRK4. Nevertheless, the preliminary data for DYRK4 is intriguing since it has been assumed that for serine/threonine kinases, it is common for there to be a preference for serine residues over threonine residues.^{13,19} It will be very interesting to explore whether DYRK4 prefers threonine residues over serine residues.

Based on our lab's unpublished data, in order to be more confident with the generated specificity motifs for DYRK2 and DYRK4, at least a few hundred unique phosphorylation sites on bacterial proteins will need to be identified for each kinase. Currently, only 77 and 23 unique phosphorylation sites have been identified for DYRK2 and DYRK4, respectively. Since our lab has identified over 4,500 unique sites for DYRK1a (*unpublished data*), we have evidence that it is possible to generate a sufficient amount of phosphorylation sites, using the ProPeL methodology, to obtain a specificity motif with a high degree of statistical significance. In order to obtain an adequate amount of unique phosphorylation sites, the ProPeL protocol will need to be repeated for DYRK2 and DYRK4.

Not to mention, minor changes may need to be made to cause these kinases work optimally. One of the challenges associated with the ProPeL protocol is that many kinases do not activate or express effectively in *E. coli*. The *Supplementary*

Materials section shows the various external conditions tested on the following protein kinases: BGLF4, CDKL5, CLK1, CLK3, DYRK2, DYRK3, and DYRK4. Different growth medias, volumes of growth media, growth temperatures, competent *E. coli* strains, induction times, incubation times, and concentrations of IPTG were tested. After testing these various growth conditions, DYRK2 and DYRK4 were the only two kinases that exhibited any kinase expression and activity (**Supplementary Figures 1 and 2**). Based on these results and previous research in our lab, it is evident that each kinase requires specific external conditions in order to fold correctly and carry out phosphorylation. Thus, additional expression conditions will need to be tested in order to generate specificity motifs for BGLF4, CLK1, CLK3, CDKL5, and DYRK3.

Future research involves using the *scan-x* tool to identify top predicted phosphorylation sites on proteins that are known to interact with DYRK2, but which presently lack specific site information.²⁹ Mutagenesis will also be implemented to identify critical residues involved in DYRK2 and DYRK4 substrate specificity relative to other DYRK family members. Using the ProPeL methodology, the Schwartz Lab aspires to determine the substrate specificity of the entire DYRK family of kinases, in the near future. The lab intends to use the substrate specificities to analyze the structure-function relationship of specificity in the CMGC clade of kinases.

V. Materials and Methods

ProPeL Methodology¹³

Molecular Cloning. The pDNR-Dual vector containing the catalytic domain of the DYRK2 (*Homo sapiens*) gene was purchased from Harvard PlasmID Repository (Plasmid ID HsCD00003955). The pDNR-Dual vector containing the catalytic domain of

the DYRK4 (*Homo sapiens*) gene was purchased from Harvard PlasmID Repository (Plasmid ID HsCD00003955).³⁰ The catalytic domain of each gene (DYRK2-AA201-550, DYRK4-AA81-420) was cloned into the pET45b bacterial expression vector (EMD Millipore) through restriction enzyme cloning and ligation. *Escherichia coli* OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 1.5mL of overnight culture was diluted into 150mL fresh media and grown under the same conditions until OD₆₀₀ reached 0.6–0.7, at which point protein expression was induced with 0.5mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown for 4 additional hours. See *Supplementary Materials* for other expression conditions. Cultures were pelleted by centrifugation at 6000g and 4°C for 15 minutes. Pellets were stored at –80°C until lysis.

Lysis and Analysis of *in vivo* Phosphorylation. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ Cell pellets were resuspended in 0.6mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). *In vivo* phosphorylation by the protein kinase of interest was

analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.

Protein Reduction, Alkylation, and Tryptic Digestion. Referring to the protocol proposed by Villén and Gygi (2008), 10mg of the protein lysate was reduced and alkylated. Then, the sample was digested overnight with recombinant trypsin (TrypZean, Sigma-Aldrich), at a 1:100 enzyme-to-substrate-ratio.³¹ Peptides were desalted with 100mg tC18 SepPak Vac solid-phase extraction cartridges (Waters), snap-frozen in liquid nitrogen, and lyophilized until the samples were a white powder.

Titanium Dioxide Phosphoenrichment. Phosphopeptide enrichment of each sample was performed using TiO₂ beads (G.L. Sciences), modified from the protocol proposed by Kettenbach *et al* (2011).³² Each sample was desalted with in-house StageTips, containing 5 C18 disks.^{13,31,33} Each sample was dried down by vacuum centrifugation and stored at -20°C.

Mass Spectrometry. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed as described by Chou *et al.* (2012).¹³ Each sample was resuspended in Buffer A (3% Acetonitrile and 0.125% formic acid in water) and injected, using a C18 nanocapillary column, directly into the inlet of an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was run using a TOP10 method (a

mass spectrometric (MS) scan followed by collision MS/MS on the 10 most intense MS spectral peaks). Each sample's spectra were analyzed using SEQUEST.^{13,34}

pLogo Analysis¹⁵

The foreground data was generated by mapping the tryptic peptides, which were mapped by MS/MS) back onto the *E. coli* protein, and extending the peptides, using the peptidextender tool, to create serine-, threonine-, or tyrosine-centered 15-mers.

Background data was obtained through aligning all of the unique serine-, threonine-, or tyrosine-centered 15-mers in the entire *E. coli* proteome.¹⁵

VI. Acknowledgements

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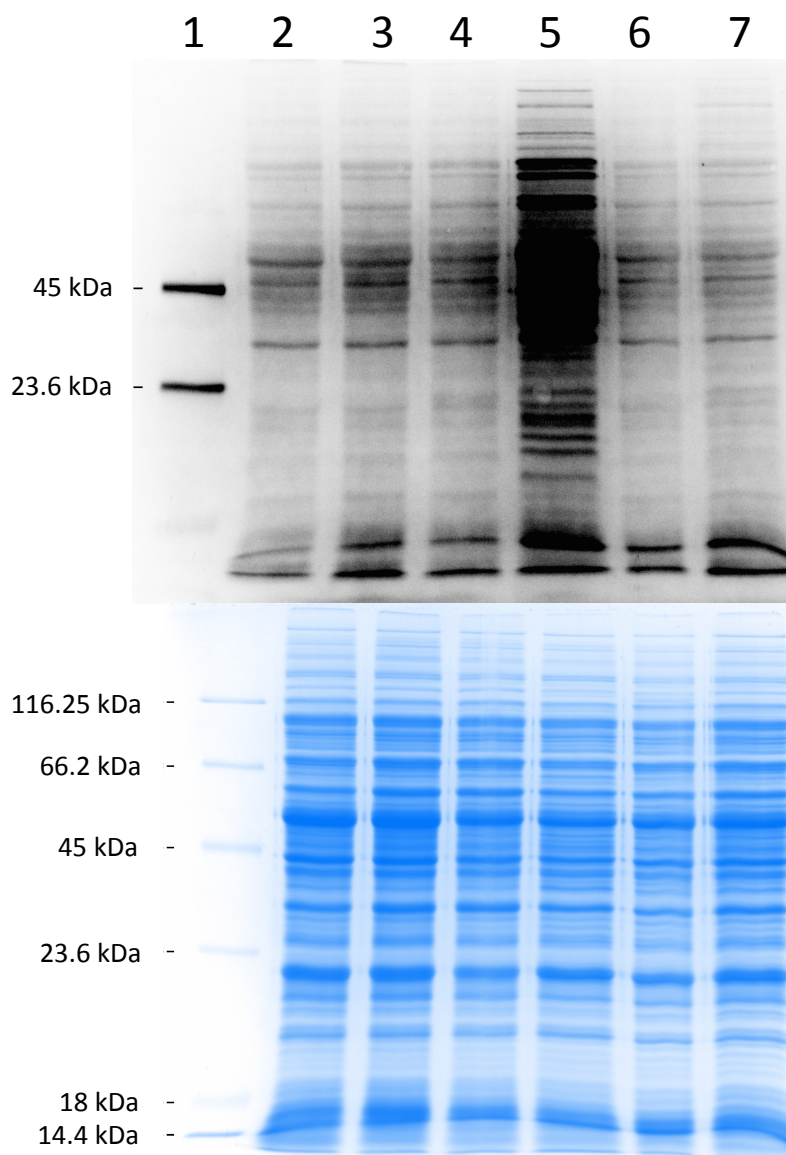
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VIII. Supplementary Material

Supplementary Table 1: Protein Kinases Analyzed in this Project					
Kinase	Plasmid ID Number	Clone Type	Number of Residues	Molecular Weight (kDa)	Activity
CDKL5	Harvard PlasmID # HsCD00022404	Catalytic Domain	320	37.01	None
CLK1	Harvard PlasmID # HsCD00005923	Full Length	484	57.40	None
CLK3	Harvard PlasmID # HsCD00002779	Catalytic Domain	357	41.86	None
DYRK2	Harvard PlasmID # HsCD00003955	Catalytic Domain	350	34.45	Expressed
DYRK3	Harvard PlasmID # HsCD00021520	Catalytic Domain	351	34.75	None
DYRK4	Harvard PlasmID # HsCD00005301	Catalytic Domain	351	32.05	Expressed
BGLF4	Addgene # 37936	Full-Length	429	48.35	None

Supplementary Table 2: Kinase Expression Conditions

Supplementary Figure #	Kinases	Cell Strain	Expression Vector	Temperature (°C)	Growth Medium	Volume of Medium (mL)	Incubation Time (hr)	Mid-log Induction (Yes/No)	IPTG Concentration (mM)	Active Kinase(s)
1	BGLF4 CDKL5 DYRK2 DYRK3 DYRK4	C41 DE3	pET45b	37	ZYM-5052	25	16	No	0	DYRK2
2	BGLF4 CDKL5 DYRK2 DYRK3 DYRK4	C41 DE3	pET45b	37	LB	25	8	No	1	DYRK2
3	BGLF4 CDKL5 DYRK2 DYRK3 DYRK4	C41 DE3	pET45b	37	LB (2-Stage Induction)	25	4 + 4	No	0.5	DYRK2
4	BGLF4 CLK1 CLK3 DYRK3 DYRK4	C41 DE3	pET45b	30	LB	25	5 (After Mid-log Induction)	Yes	0.5	DYRK4
5 (Same as Figure 5)	DYRK2 DYRK4	C41 DE3	pET45b	30	LB	150	4 (After Mid-log Induction)	Yes	0.5	DYRK2 DYRK4
6 (Lanes 2-5)	DYRK2 DYRK3 DYRK4	Rosetta	pET45b	37	LB	25	16 (After Mid-log Induction)	Yes	0.5	DYRK2
6 (Lanes 6-9)	DYRK2 DYRK3 DYRK4	Rosetta	pET45b	20	LB	25	16 (After Mid-log Induction)	Yes	0.5	DYRK4



Supplementary Figure 1

Top: Pro-Q® Diamond Phosphoprotein Gel Stain

Bottom: GelCode™ Blue Safe Protein Stain

Lane 1: PeppermintStick™ Phosphoprotein Molecular Weight Standards

Lane 2: pET45b (Empty Vector Control)

Lane 3: BGLF4

Lane 4: CDKL5

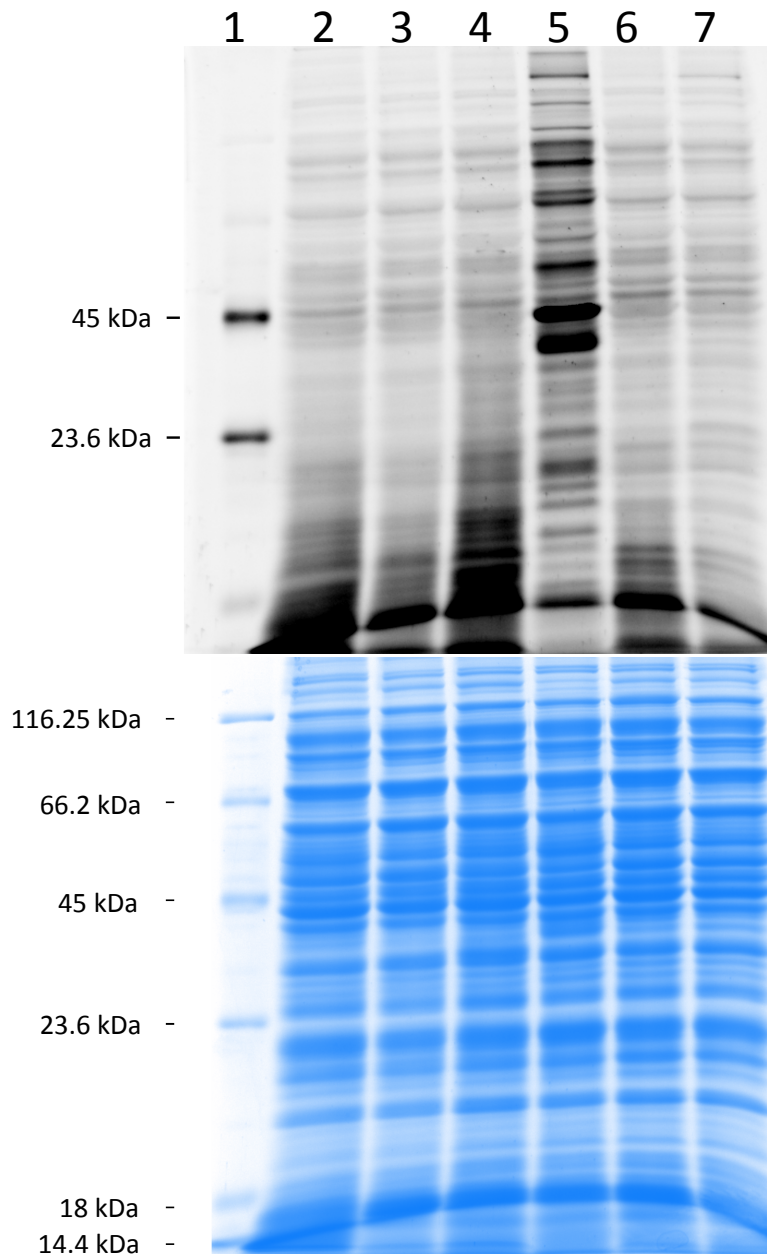
Lane 5: DYRK2

Lane 6: DYRK3

Lane 7: DYRK4

Materials and Methods: Supplementary Figure 1

Escherichia coli OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of ZYM-5052 (auto-inducing medium),³⁵ supplemented with 100µg/mL of ampicillin, at 37°C and 250rpm for 16 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). *In vivo* phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.



Supplementary Figure 2

Top: Pro-Q® Diamond Phosphoprotein Gel Stain

Bottom: GelCode™ Blue Safe Protein Stain

Lane 1: PeppermintStick™ Phosphoprotein Molecular Weight Standards

Lane 2: pET45b (Empty Vector Control)

Lane 3: BGLF4

Lane 4: CDKL5

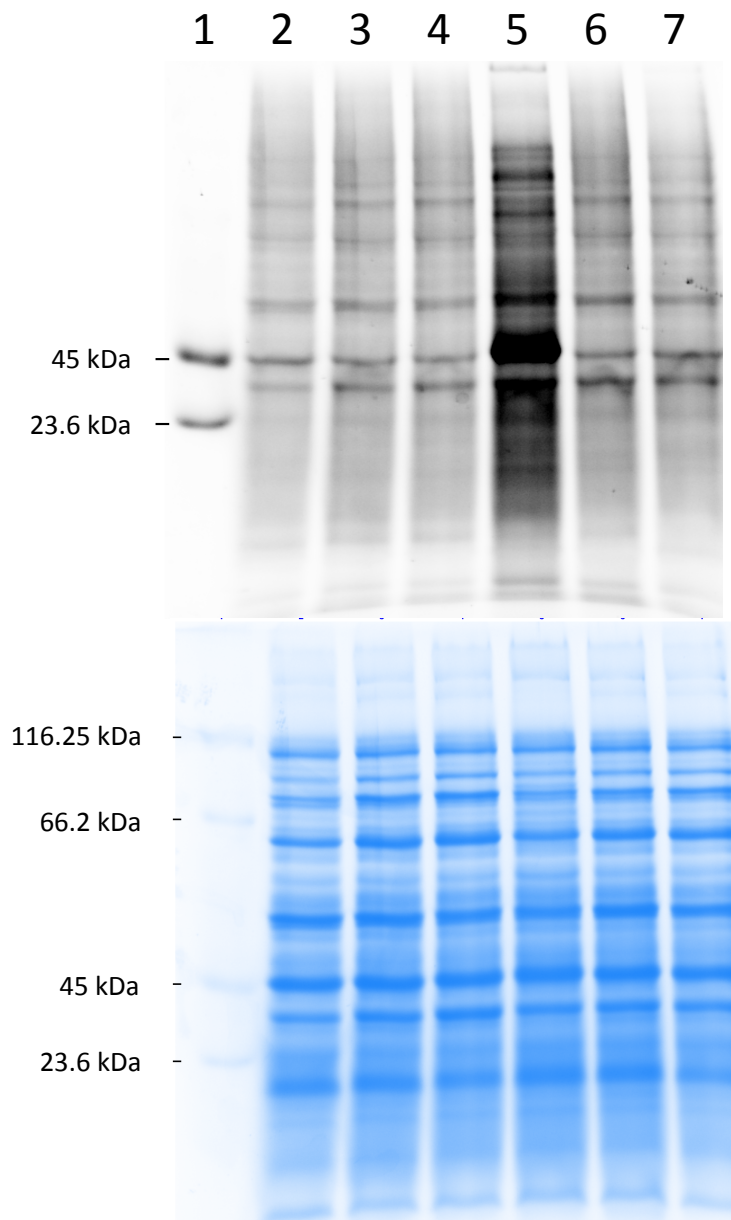
Lane 5: DYRK2

Lane 6: DYRK3

Lane 7: DYRK4

Materials and Methods: Supplementary Figure 2

Escherichia coli OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of LB broth, supplemented with 100µg/mL of ampicillin and 1mM IPTG, at 37°C and 250rpm for 8 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.

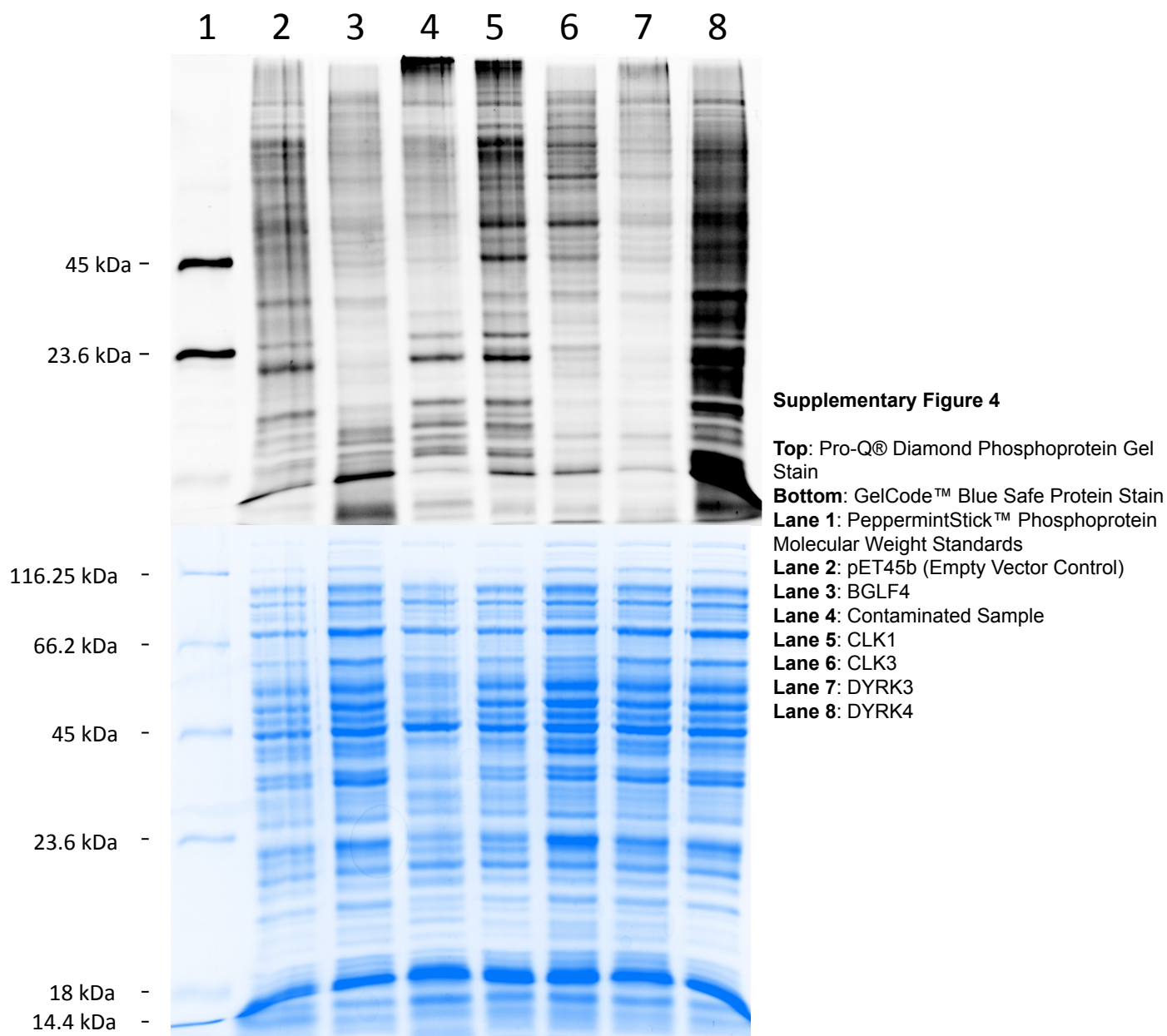


Supplementary Figure 3

Top: Pro-Q® Diamond Phosphoprotein Gel Stain
Bottom: GelCode™ Blue Safe Protein Stain
Lane 1: PeppermintStick™ Phosphoprotein Molecular Weight Standards
Lane 2: pET45b (Empty Vector Control)
Lane 3: BGLF4
Lane 4: CDKL5
Lane 5: DYRK2
Lane 6: DYRK3

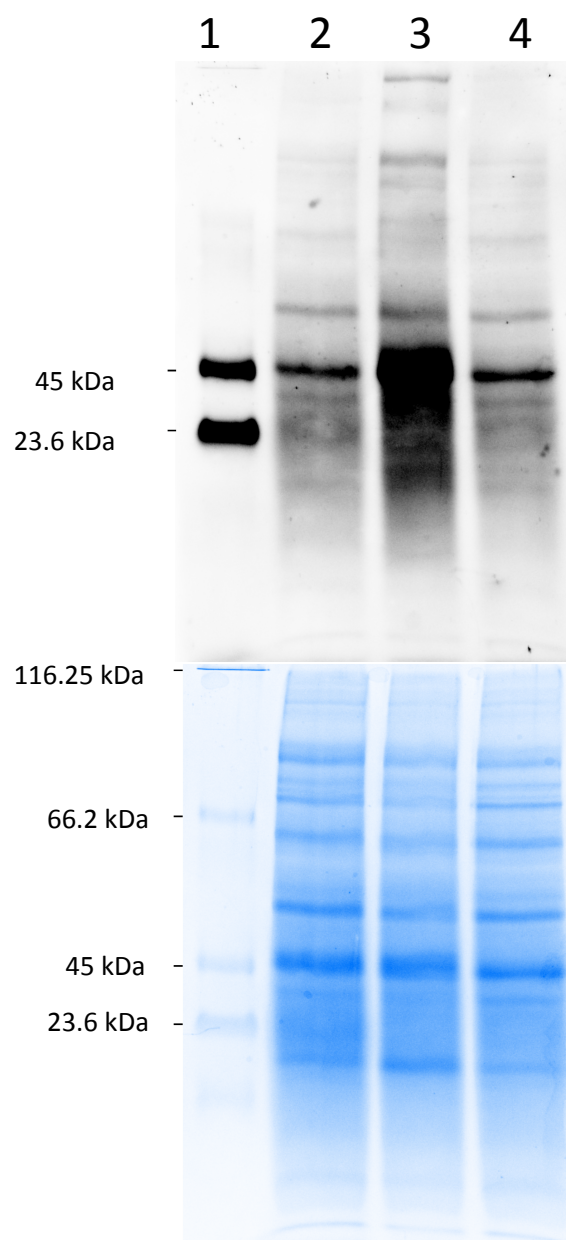
Materials and Methods: Supplementary Figure 3

Escherichia coli OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of LB broth, supplemented with 100µg/mL of ampicillin and 0.5mM IPTG, at 37°C and 250rpm for 4 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C and washed twice with 10mL of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·12H₂O, 1.8 mM KH₂PO₄; pH 7.3), then centrifuged again. The washed cell pellet was resuspended thoroughly in 25 mL of fresh LB broth supplemented with 100µg/mL ampicillin and 0.5mM IPTG. The culture was grown at 37°C and 250rpm for 4 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.



Materials and Methods: Supplementary Figure 4

Escherichia coli OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of LB broth, supplemented with 100µg/mL of ampicillin, at 30°C and 250rpm. The culture was induced at mid-log stage with 0.5mM IPTG and grown for an additional 5 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.



Supplementary Figure 5

Top: Pro-Q® Diamond Phosphoprotein Gel Stain

Bottom: GelCode™ Blue Safe Protein Stain

Lane 1: PeppermintStick™ Phosphoprotein Molecular Weight Standards

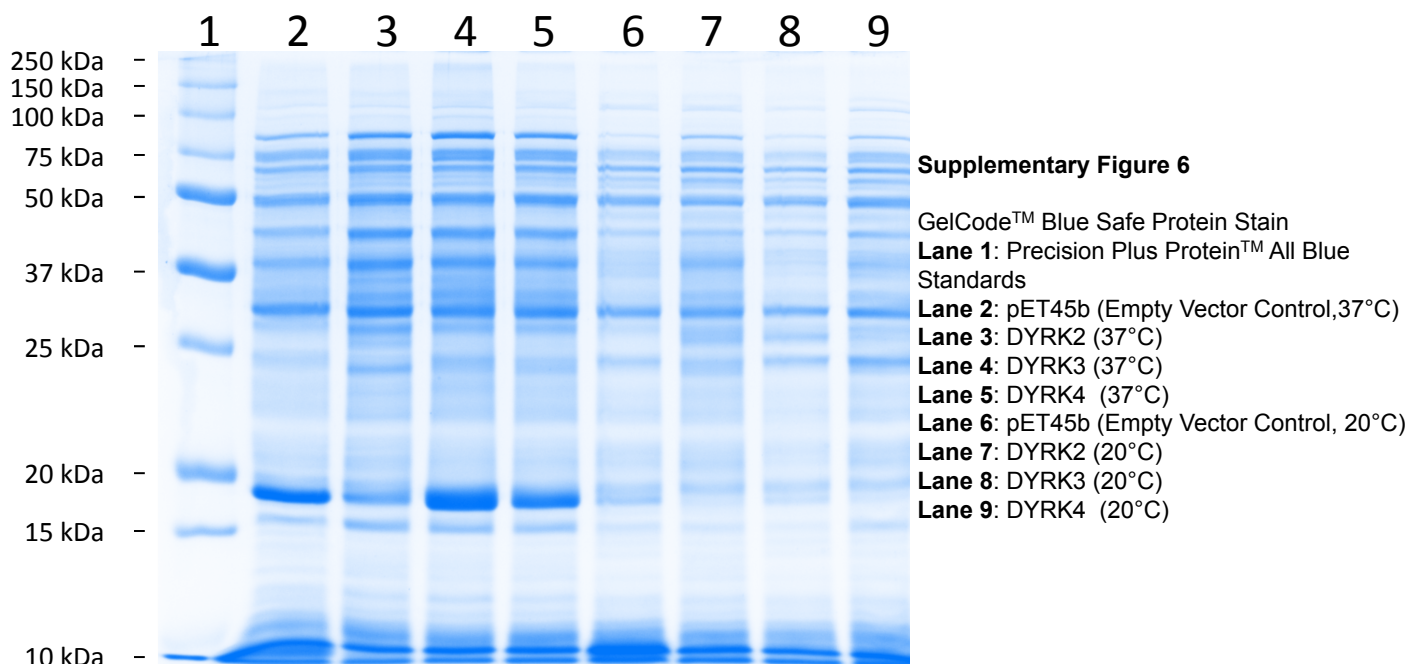
Lane 2: pET45b (Empty Vector Control)

Lane 3: DYRK2

Lane 4: DYRK4

Materials and Methods: Supplementary Figure 5

Escherichia coli OverExpress C41(DE3) cells (Lucigen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 1.5mL of overnight culture was diluted in 150mL of LB broth, supplemented with 100µg/mL of ampicillin, at 30°C and 250rpm. The culture was induced at mid-log stage with 0.5mM IPTG and grown for an additional 4 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.6mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was first stained with Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies), which specifically stains phosphoproteins in polyacrylamide gels. GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific) was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.



Materials and Methods: Supplementary Figure 6 (Lanes 2-5)

Escherichia coli Rosetta™ competent cells (Novagen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 37°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of LB broth, supplemented with 100µg/mL of ampicillin, at 37°C and 250rpm. The culture was induced at mid-log stage with 0.5mM IPTG and grown for an additional 16 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific), which was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.

Materials and Methods: Supplementary Figure 6 (Lanes 6-9)

Escherichia coli Rosetta™ competent cells (Novagen) were transformed with the pET45b bacterial expression vector containing the gene-of-interest via heat shock treatment. The cells were plated on Luria-Bertani (LB) plates supplemented with 100µg/mL ampicillin. Colonies were inoculated in LB broth supplemented with 100µg/mL ampicillin and grown up overnight in a shaking incubator set at 20°C and 250rpm. 0.5mL of overnight culture was diluted in 25mL of LB broth, supplemented with 100µg/mL of ampicillin, at 20°C and 250rpm. The culture was induced at mid-log stage with 0.5mM IPTG and grown for an additional 16 hours. The cells were harvested by centrifugation at 6000g for 15 minutes at 4 °C. Cell lysate was prepared according to the protocol proposed by Villén and Gygi (2008), with a few modifications.³¹ The cell pellets were resuspended in 0.4mL of lysis buffer (8M urea, 75mM NaCl, 60mM Tris, pH 8.2) supplemented with Halt Protease Inhibitor Cocktail (EDTA-free) and Halt Phosphatase Inhibitor Cocktail (Pierce). The cells were lysed, using a sonicator set at 15% power. 5 rounds of 15-second pulses, with a 1-minute rest on ice between pulses, were sufficient for complete lysis. Crude lysate was clarified by centrifugation at 20,000g and 4°C for 30 minutes. Protein concentrations were determined by Bichinchoninic Acid (BCA) Assay (Pierce). In vivo phosphorylation by the protein kinase of interest was analyzed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size. The gel was stained with GelCode™ Blue Safe Protein Stain (Thermo Fisher Scientific), which was utilized to evaluate the total protein made from induction. The remaining protein lysates were stored at -80°C.